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Enhanced positive selection of a transgenic TCR by a restriction element that does not permit negative selection

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Abstract

Very little is known about the conformational properties of the MHC molecules that are able to signal positive selection of a given TCR. To try to understand these parameters and to determine whether these requirements are shared with interactions during negative selection and antigen recognition, we have studied selection and antigen recognition of a transgenic TCR (specific for lymphocytic choriomeningitis virus glycoprotein and H-2D^b) in the context of two D^b mutants, H-2^{bm13} and H-2^{bm14}. The data showed that the transgenic TCR was not positively selected by the H-2^{bm14} haplotype but, interestingly, enhanced positive selection was seen in H-2^{bm13} mice. The transgenic TCR could not be negatively selected in H-2^{bm13} animals persistently infected with the virus (neonatal virus carrier mice), nor could the transgenic TCR be activated by H-2^{bm13} infected cells *in vivo* or *in vitro*. These experiments show that although a TCR may be selected by a mutant MHC molecule, the corresponding viral antigen cannot be recognized in the context of the mutant MHC molecule, as judged by both negative selection and T cell reactivity *in vivo* and *in vitro*. The 'enhanced' positive selection occurring in the context of D^{bm13} suggests that a different conformation of the MHC molecule is able to select the same TCR and also that various TCR – ligand avidities may permit positive selection.

Introduction

The TCR expressed on T lymphocytes is able to transmit signals for three different developmental or functional events: positive selection, tolerance induction, and activation by antigen. The stringent requirements for TCR – ligand interaction have been well characterized for antigen recognition indicating that a defined peptide is presented by a defined MHC molecule with limited variability (for a review see 1). In several systems certain amino acid residues of the peptide antigen (2,3) or of the MHC molecule (4 – 7) have been shown to be essential for T cell recognition (8 and reviewed in 9). In general, TCR recognition of a given peptide presented by a particular MHC is relatively strict.

Several studies have dealt with the interactions that are able to signal negative selection. Studies using superantigens such as Mls and Staphylococcal enterotoxin B (SEB) have shown that a 'gradient' of the clonal deletion may be defined depending on the presenting haplotype (10 – 13). Using a TCR transgenic model specific for the alloantigen H-2L^d, Sha *et al.* (14) have shown that a single amino acid change in the K^b molecule, which is required for positive selection, resulted in the deletion of T cells. In general, however, it is difficult to judge the flexibility of the interacting ligands during negative selection in classic antigen – MHC-restricted recognition. Recently, Pircher *et al.* (15) showed that a variant peptide that is able to induce clonal deletion of T cells expressing a transgenic receptor is not able to activate the transgenic T cells. This suggests that antigen recognition by the TCR is demanding higher avidity interactions than tolerance induction.

Other studies have evaluated the TCR – ligand interactions during positive selection and suggest that a peptide may be involved in positive selection (14,16 – 19). Using TCR transgenic mice restricted to D^b, Jacobs *et al.* (18) showed that positive selection does not occur in the presence of D^{bm13} and D^{bm14}.

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Sha *et al.* (14) demonstrated that a transgenic K^b-restricted receptor cannot be positively selected in three of the four K^{bm} mutants that could be analysed, suggesting that the interaction between the TCR and the selecting complex is relatively rigid.

Based on these experiments, the precise fit, flexibility, and conformational requirements (i.e. the avidity) of a given TCR – MHC – peptide interaction during positive selection, negative selection, and antigen activation is difficult to define at present. Therefore a lymphocytic choriomeningitis virus (LCMV)-D^b-specific transgenic TCR for positive and negative selection *in vivo*, as well as for antigen recognition *in vivo* and *in vitro*, in mice expressing mutant D^b molecules has been evaluated.

Methods

Animals

Inbred mice were purchased from the Institut für Zuchthygiene, Tierspital, University of Zurich, Switzerland. B6.C-H-2^{bm13} (H-2^{bm13}) and B.6C-H-2^{bm14} (H-2^{bm14}) animals (20,21) were kindly provided by Dr Cornelis J. Melief. The TCR transgenic mouse line 327 (22) was crossed into the H-2^{bm13} and H-2^{bm14} strains using the following strategy. TCR transgenic H-$2^{b/d}$ animals were developed by mating the TCR transgenic H-$2^{b/b}$ animals with BALB/c mice (H-$2^{d/d}$). It has been previously demonstrated that this transgenic TCR could not be positively selected in a predominantly BALB/c background (23). TCR transgenic positive H-$2^{b/d}$ animals were then bred with homozygous H-2^{bm13} or H-2^{bm14} animals. Offspring from these breeding pairs were either H-$2^{b/bm}$ or H-$2^{d/bm}$ that were positive or negative for the transgene. The MHC haplotype was determined by staining peripheral blood lymphocytes with an H-2^d-specific antibody (24).

Flow cytometry analysis

Cells (1×10^6) were incubated with various mAbs in 100 μl volume balanced salt solution (BSS); 2% fetal calf serum (FCS); 0.1% sodium azide for 30 min at 4°C. The cells were washed and incubated with fluoresceinated antibodies. Analysis of blood lymphocytes was performed after lysis of red blood cells. Viable cells (10,000) were analysed on an EPICS profile analyser for single parameter analysis and 20,000 viable cells were analysed in 2 – 3-colour histograms. The mAbs used were KT3 (rat anti-CD3; 25), KJ16 (rat anti-V_β8.1, 8.2; 26), B20.1 (rat anti-V_α2; 27), and anti-CD4 conjugated with phycoerythrin (PE) (Becton-Dickinson, Belgium), FITC conjugated anti-CD8 (Becton-Dickinson). For 3-colour analysis, biotinylated anti-CD8 was used together with streptavidin-RED613 (Gibco BRL, Basel, Switzerland). The percentages given for 2-colour analysis were obtained by defining quadrants based on the staining of thymocytes from negative littermate controls. The figures have been cropped to show the relevant data.

Cytotoxic assays

Animals were primed with 200 p.f.u. of the WE strain of LCMV (obtained from Dr F. Lehmann-Grube, Hamburg, Germany). Eight days later the spleen was removed and the effector cells were incubated, together with target cells, at a ratio of 70:1, 23:1, 8:1, and 3:1. Target cells were either fibroblast lines MC57G (K^bD^b), B10.A(5R) (K^bD^d), or B10.HTG (K^dD^b), or concanavalin A (Con A) induced spleen cell blasts. The blast cells were prepared by culturing spleen cells with 3 μg/ml Con A (Pharmacia, Uppsala, Sweden) for 48 h and purification over Ficoll gradients. Targets were incubated with ^{51}Cr, with or without peptides, for 2 h. The peptide antigen used in these experiments was IKAVYNFATCG. The effector cells were incubated with target cells for 4 – 5 h and the supernatant was removed and counted. Per cent specific release was calculated as described previously (28).

Proliferation assays

Lymph node cells (1×10^5) from TCR transgenic H-2^b mice and from negative littermate controls were incubated with 2×10^4 LCMV-infected macrophages or uninfected macrophages from H-2^b and H-2^{bm13} mice. Stimulator macrophages were irradiated with 2000 rad. After 48 h [^{3}H]thymidine was added overnight. The wells were harvested and incorporated radioactivity was counted. Data show the average counts from triplicate samples.

Generation of virus carrier animals

Newborn mice from (H-2^d transgenic × H-2^{bm}) F_1 or control H-2^b transgenic mice were injected with 3×10^5 p.f.u. of LCMV i.p. Mice (3- to 4-week-old) were sacrificed and thymocytes were analysed by flow cytometry. Mice were confirmed to be virus carriers by injection of 30 μl of their blood into the footpad of mice. Footpad swelling was seen 8 – 9 days after challenge with the blood, indicating that the virus could be transferred from the carrier animals.

Results

H-2D^b is required for positive selection of the transgenic TCR

TCR transgenic mice have been generated from a cytotoxic T cell clone specific for the LCMV glycoprotein (GP) (amino acids 32 – 42) presented in the context of D^b (22). To formally demonstrate that the TCR transgenic receptor requires the D^b molecule for positive selection, H-2^d TCR transgenic mice were bred with B10.HTG (K^dD^b) and B10.A(5R) (K^bD^d) mice. Positive selection of class I restricted transgenic TCRs has previously been characterized by a skewing of thymocytes to the CD8 lineage (22,29,30). Thymocytes from TCR transgenic B10.HTG and B10.A(5R) animals were stained with CD4 and CD8 and compared with TCR transgenic animals in the selecting H-2^b (19.0% $CD8^+$ ± 2.4, n = 7) and non-selecting H-2^d background (6.0% $CD8^+$ ± 1.4, n = 4) (Fig. 1). Prominent skewing to the CD8 lineage was seen only in the presence of D^b (24.5% $CD8^+$ ± 0.7, n = 2). Further analysis has shown that the transgenic α and β TCR are present at high levels on $CD8^+$ cells only in animals expressing H-2^b and not H-2^d (31,32). Taken together, these data show that positive selection of the transgenic TCR occurs only in the presence of D^b.

The effects of changes in the groove of the MHC or TCR – MHC interaction during positive selection, negative selection, and antigen recognition was examined using two D^b mutant mouse strains called B6.C-H-2^{bm13} (H-2^{bm13}; K^bD^{bm13}) and B6.C-H-2^{bm14} (H-2^{bm14}; K^bD^{bm14}) (20). H-2^{bm13} has three amino acid substitutions on the β-pleated sheet of the D^b class I molecule (residues 114 Leu – Glu, 116 Phe – Tyr, 119 Glu – Asp), while H-2^{bm14} has a single amino acid change on the α-helix of D^b (residue 70 Glu – Asp) (21). These alterations of the D^b molecule result in

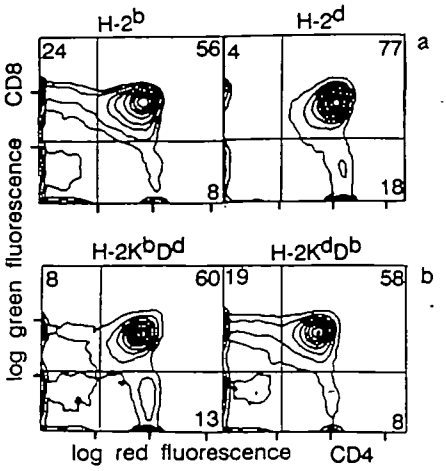

Fig. 1. Positive selection of the transgenic TCR by D^b. Thymocytes from TCR transgenic mice (K^dD^d, BALB/c origin) bred with C57BL/6 (K^bD^b), B10.A(5R)(K^bD^d), and B10.HTG (K^dD^b)] were analysed using mAbs specific for CD4 and CD8. At least two animals were analysed in each haplotype and a representative analysis is shown. The following percentages were obtained. K^bD^b: 19.0% $CD8^+$ ± 2.4, 63% $CD4^+CD8^+$ ± 6.6, 4.6% $CD4^+$ ± 2.5, n = 7; K^dD^d: 6.0% $CD8^+$ ± 1.4, 69.0% $CD4^+CD8^+$ ± 10.9, 15.8% $CD4^+$ ± 5.5, n = 4; K^bD^d: 9.5% $CD8^+$ ± 2.2, 54.5% $CD4^+CD8^+$ ± 6.3, 22% $CD4^+$ ± 5.6, n = 2; K^dD^b, 24.5% $CD8^+$ ± 0.7, 56.0% $CD4^+CD8^+$ ± 0.0, 8% $CD4^+$ ± 0.0, n = 2.

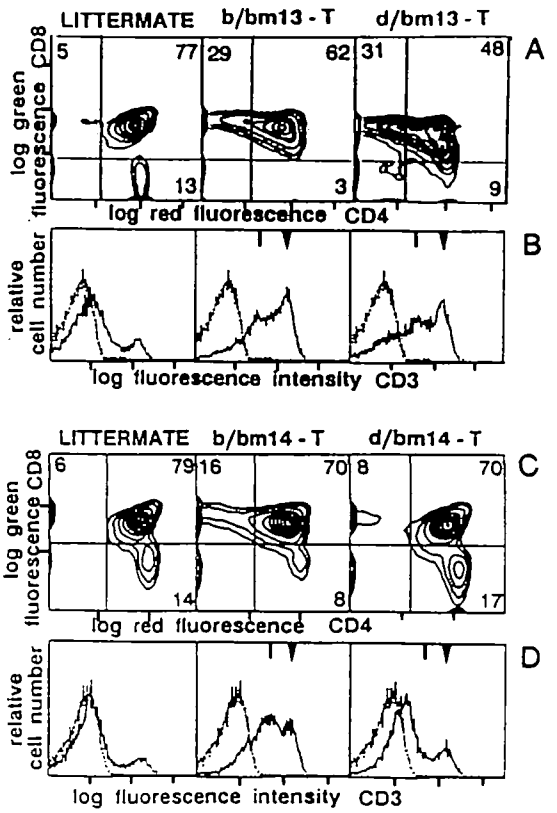

Fig. 2. Positive selection of the transgenic TCR by H-2^{bm13} but not H-2^{bm14}. Thymocytes from H-$2^{b/bm}$ and H-$2^{d/bm}$ transgenic mice, as well as nontransgenic littermate controls, were analysed in the H-2^{bm13} (A and B) and H-2^{bm14} (C and D) mutant phenotypes using mAbs specific for CD4, CD8 and CD3 (KT3) (25). (B and D) Broken lines represent cells stained with the second stage antibody alone. The vertical bar at the top of the profiles indicates the TCR – CD3 intermediate population and the triangle indicates TCR – $CD3^{hi}$ cells. Four to six animals of each type were analysed and a representative analysis is shown. The following percentages were obtained. K^bD^{bm13}: 32.0% $CD8^+$ ± 3.4, 44.3% $CD4^+CD8^+$ ±10.7, 13.9% $CD4^+$ ± 7.5, n = 6; K^bD^{bm14}: 7.8% $CD8^+$ ± 1.0, 60.7% $CD4^+CD8^+$ ±10.1, 14.2% $CD4^+$ ± 7.8, n = 4.

changes that have been predicted to alter the peptide binding pocket, as determined by computer modelling (33).

H-2D^{bm13} permits positive selection of the transgenic TCR

Positive selection of the transgenic TCR was studied in H-2^{bm13} and H-2^{bm14} mice using two criteria: a skewing to $CD8^+$ thymocytes (22,29,30) and the presence of a subpopulation of thymocytes expressing CD3 at intermediate levels which has been previously correlated with positive selection (23,34,35). Because positive selection of the transgenic TCR does not occur in H-2^d mice, H-$2^{d/bm}$ mice were examined for the positive selection of the transgenic TCR. TCR transgene positive H-$2^{b/bm}$ mice were examined as controls to ensure that the D^{bm} alleles did not negatively affect maturation of the TCR transgene positive thymocytes. Thymocytes stained with CD4 and CD8 revealed skewing of the transgenic TCR towards $CD8^+$ thymocytes in H-$2^{d/bm13}$ mice (32.0% $CD8^+$ ± 3.4%, n = 6) but not H-$2^{d/bm14}$ mice (7.8% $CD8^+$ ± 1.0%, n = 4) (Fig. 2A and C). An unusual population of $CD4^+CD8^{lo}$ cells was often seen in H-$2^{d/bm13}$ mice, but how they fit into T cell ontogeny in these animals is not known. CD3 single parameter analysis of thymocytes showed the presence of TCR $CD3^{int}$ peaks in mice carrying the H-2^b allele or in H-$2^{d/bm13}$, but not the H-$2^{d/bm14}$ TCR transgenic mice (Fig. 2B and D). Comparable results are also seen when the transgenic V_α- or V_β-specific antibodies were used for analysis (data not shown). These results indicated that positive selection occurred in H-2^{bm13}, but not H-2^{bm14}, mice.

Enhanced positive selection by H-2^{bm13}

Interestingly examination of positive selection of the transgenic TCR by D^{bm13} compared with D^b consistently showed a more prominent skewing to the $CD8^+$ thymocyte population (Fig. 3A). In D^b animals the $CD8^+$ population in the thymus is 19.0% (± 2.4%, n = 7). The $CD8^+$ thymocyte population in D^{bm13} animals is ~34.0% (± 3.4%, n = 6). In addition, an increased proportion of TCR $CD3^{hi}$-bearing cells was seen, compared with the number of T cells expressing the TCR CD3 at intermediate densities (Fig. 3B). Examination of TCR transgenic thymocytes in the presence of D^b and D^{bm13} by 3-colour cytofluorometric analysis was also performed to study the TCR CD3 levels during the progression from double positive thymocytes to $CD8^+$ thymocytes. In the $CD8^+$ thymocytes, a high level of CD3 expression was seen, as expected in a mature subpopulation of T cells in both the D^b and D^{bm13} transgenic animals (Fig. 4a). The $CD4^{lo}/CD8^+$ subset contained a majority of $CD3^{int}$ in the D^b transgenic animals, compared with the predominant $CD3^{hi}$ cells in the D^{bm13} mice (Fig. 4b). The $CD4^+CD8^+$ population contained very few $CD3^{hi}$ cells in D^b mice, while the D^{bm13} double positive thymocytes had considerably more $CD3^{hi}$ cells

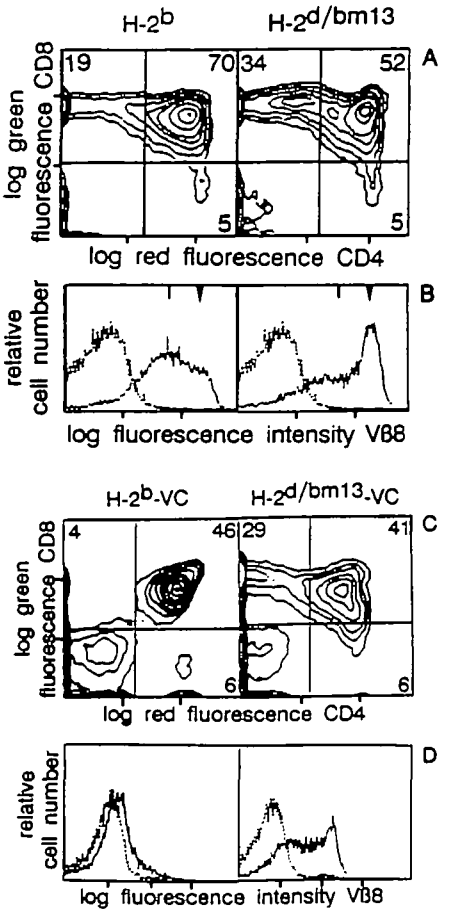

Fig. 3. Positive and negative selection of the transgenic TCR in mice carrying the H-2^{bm13} alleles. (A) Thymocytes from H-2^{b} TCR transgenic mice or TCR transgenic H-$2^{d/bm13}$ mice were compared using CD4 and CD8 (B) or KJ16 that reacts with the transgenic receptor $V_{\beta}8.1$ (26). (C and D) Clonal deletion of the transgenic TCR was examined in H-2^{b} and H-2^{bm13} virus carrier mice. (C) Thymocytes from 3- to 4-week-old mice were analysed with CD4 and CD8 or (D) KJ16 mAbs. Profiles for (B) and (D) are as described in Fig. 2. A representative animal chosen from the three analysed is shown in the figure.

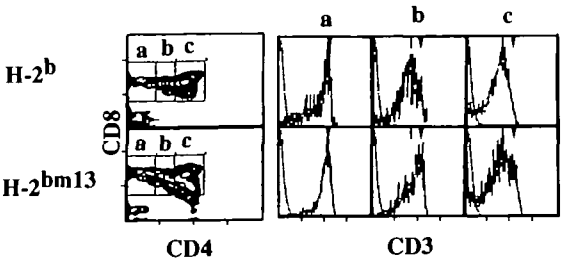

Fig. 4. Comparison of TCR – CD3 expression from double positive to single CD8 positive thymocytes in H-2^{b} and H-2^{bm13} transgenic mice. Thymocytes from H-2^{b} and H-2^{bm13} were triple stained with CD3, CD4, and CD8 antibodies. The CD4CD8 profile is shown (left), and parts a, b, and c (right) show the levels of CD3 on thymocytes expressing different levels of CD4 and CD8, as shown in the corresponding boxed areas a, b, and c. (a) CD3 expression on $CD8^{+}$ thymocytes; (b) CD3 expression on $CD4^{lo}CD8^{+}$ thymocytes; and (c) CD3 expression on $CD4^{+}CD8^{+}$ thymocytes. The vertical bar indicates thymocytes expressing TCR – CD3 at intermediate levels and the triangle indicates TCR – CD3 high levels. It should be noted that in these animals CD3 expression parallels the expression of the transgenic TCR as assessed by staining with KJ16 and B20.1 V region-specific antibodies (23 and data not shown). Two animals have been examined and similar results were obtained.

Table 1. Analysis of T cells in the blood and lymph node

Mice		Blood		Lymph node cells	
		%CD3	%CD3	%CD4	%CD8
H-2^{b}	tg^{-}	32 ± 5	55 ± 4	38 ± 1	18 ± 3
	tg^{+}	36 ± 4	59 ± 4	11 ± 2	57 ± 7
H-2^{bm13}	tg^{-}	31 ± 2	55 ± 3	39 ± 2	19 ± 5
	tg^{+}	52 ± 4	68 ± 5	7 ± 1	69 ± 5

(Fig. 4c). This suggested that the transgenic TCR may be more efficiently selected to express TCR CD3 at high levels in the H-2^{bm13} haplotype.

To examine whether efficient selection resulted in an increase of peripheral T cells, the number of T lymphocytes in the blood and lymph nodes was investigated. The peripheral blood lymphocytes from TCR transgenic H-2^{bm13} mice had an increased number of T cells (52 ± 4%, n = 8) compared with transgene negative littermate controls (31 ± 2%, n = 8) or TCR transgenic H-2^{b} mice (36 ± 4%, n = 8) (Table 1). Lymph nodes also contained elevated levels of T cells, with a predominant skewing to $CD8^{+}$ cells in both D^{b} and D^{bm13} transgenic animals (Table 1). Transgenic H-2^{bm13} mice, ranging in age from 20 days to 6 months, show similar distributions of CD3, CD4, and CD8 positive cells. Analysis of the expression of the transgenic TCR on $CD8^{+}$ lymph node cells was also performed (Fig. 5). Two-colour anlaysis, using either B20.1 ($V_{\alpha}2$) and CD8 or KJ16 ($V_{\beta}8$) and CD8, indicate that virtually all $CD8^{+}$ cells express the transgenic V_{α} and V_{β}. Taken together these data suggest that D^{bm13} was able to more efficiently select the transgenic TCR compared with the original D^{b} molecule. It is possible that the increase in T cells is also caused by peripheral expansion of T cells expressing the transgenic receptor.

Clonal deletion of the transgenic TCR does not occur in H-2^{bm13} mice neonatally infected with LCMV

Negative selection of LCMV-specific transgenic T cells was examined in transgenic H-2^{bm13} animals. Previous experiments have shown that injection of newborn TCR transgenic mice with LCMV results in tolerance to the virus by clonal deletion of the $CD4^{+}CD8^{+}$ double positive TCR transgenic cells in H-2^{b} mice (22), but not H-2^{d} mice (data not shown). To determine if clonal deletion of the transgenic TCR also occurs in H-2^{bm13}, H-2^{d} TCR transgenic mice were bred with H-2^{bm13} mice and offspring were neonatally infected with LCMV. Thymocytes were examined by 2-colour cytofluorometry at 3 – 4 weeks of age using anti-CD4, anti-CD8, and V_{β}-specific mAbs. Thymocytes from H-$2^{d/bm13}$ TCR transgenic carrier mice showed a skewing to the $CD8^{+}$ mature subset (Fig. 3C). Analysis with $V_{\beta}8$-specific antibodies showed that the transgenic TCR was expressed on the majority of H-$2^{d/bm13}$ thymocytes at intermediate and high levels,

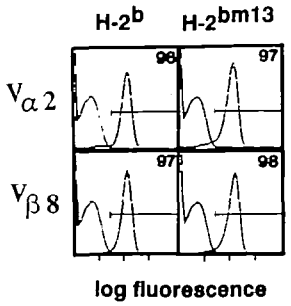

Fig. 5. Transgenic TCR expression on CD8+ lymph node cells. Lymph nodes cells from TCR transgenic H-2^b and H-2^{bm13} animals were stained with B20.1 and CD8 or KJ16 and CD8. The $V_\alpha 2$ expression (B20.1) or $V_\beta 8$ expression (KJ16) on CD8+ T cells is shown in the figure. This figure is representative of three animals.

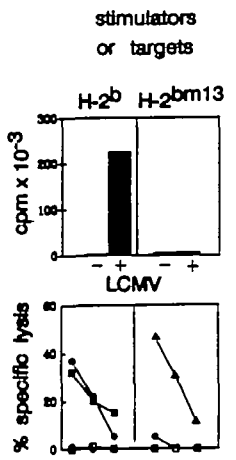

Fig. 6. Transgenic TCR does not recognize LCMV presented in the context of H-2^{bm13}. Proliferation assays (top panel) using unprimed transgenic lymph node cells and either infected (+) or uninfected (−) macrophages from H-2^b or H-2^{bm13} animals were performed. For lower values the SEM was between 30 and 50 c.p.m., while the positive response had an SEM of 10.4 × 10^{-3}. Lymph node cells from transgene negative littermates were used as negative controls. The thymidine incorporation from these wells ranged from 300 to 600 c.p.m. (not shown in this figure). This figure is representative of four animals. Cytotoxicity assays (bottom panel) using effector spleen cells from H-2^b TCR transgenic mice (●), H-2^{bm13} (▲), or C57BL/6 mice (■) primed with LCMV. Spleen cell blasts from the H-2^b or H-2^{bm13} mice were used as target cells and labelled with ^{51}Cr with (●, ■) or without (○,□) the GP peptide (32 – 42). Effector to target ratios began at 70:1, with 3-fold dilutions.

suggesting that clonal deletion did not occur in H-2^{bm13} transgenic carrier mice (Fig. 3D). Also, 80 – 90% of peripheral CD8+ T cells were found to express both transgenic V_α and V_β receptors (data not shown). This suggests that either LCMV determinants presented together with H-2^{bm13} were not recognized by the transgenic TCR thymocytes and/or that the glycoprotein peptide is not efficiently presented by D^{bm13} *in vivo*.

Transgenic T cells are not activated by LCMV-infected D^{bm13} cells

Experiments were performed to see whether mature peripheral transgenic TCR could recognize LCMV-infected H-2^{bm13} cells. When T cell competent mice are infected intracerebrally with LCMV, they succumb to a CD8 T cell mediated immunopathological disease. However, if mice cannot mount a strong antiviral cytotoxic T lymphocyte (CTL) response at the optimal time after infection, the mice may survive (36 – 38). To determine whether antigen recognition occurs *in vivo*, TCR transgenic H-$2^{d/bm13}$ and H-$2^{b/bm13}$ mice were injected intracerebrally with 2×10^6 p.f.u. of LCMV. The majority of transgenic mice that expressed the H-2^b allele died within 3 – 7 days (5/6, 4 ± 1.7), while none of the H-$2^{d/bm13}$ (0/4) transgenic animals died. This indicated that the transgenic TCR cannot efficiently recognize LCMV-infected H-2^{bm1} cells *in vivo*.

The ability of the transgenic receptor to recognize LCMV-infected or peptide-coated H-2^{bm13} cells was also evaluated by two *in vitro* assays. Proliferation assays were performed by culturing TCR transgene positive lymph node cells (H-2^b) with uninfected or LCMV-infected macrophages isolated from H-2^b or H-2^{bm13} mice. Lymph node cells from non-transgenic littermates were used as negative control cells. Proliferation measured by thymidine uptake was seen only when transgenic lymph node cells were stimulated with LCMV-infected H-2^b macrophages (Fig. 6), but not infected H-2^{bm13} macrophages. CTL assays, using effector cell populations from TCR transgenic mice, C57BL/6, and H-2^{bm13} mice primed with LCMV, were tested on H-2^b or H-2^{bm13} target cells that were either preincubated for 2 h with or without the peptide GP(32 – 42). Figure 6 indicates that transgenic effector cells could only recognize target cells expressing the GP peptide plus H-2^b, and not the peptide presented in the context of H-2^{bm13}. Taken together, these experiments demonstrate that the transgenic TCR cannot recognize LCMV-infected H-2^{bm13} cells.

H-2^{bm13} and peptide GP(32 – 42) presentation

Results from the CTL assay showed that H-2^{bm13} mice (K^bD^{bm13}) could generate a CTL response specific for the peptide GP(32 – 42). To determine whether the cytotoxic response was mediated by peptide GP(32 – 42) binding to K^b or D^{bm13}, the following assays were performed, based on the assumption that D^b-restricted effector T cells would cross react with the GP peptide presented on D^{bm13} and vice versa. Cross reactivity of D^b-restricted clones on D^b mutants expressing the appropriate antigen has been previously demonstrated (4,5), and therefore an *in vivo* polyclonal response could possibly generate a cross reactivity D^b- or D^{bm13}-restricted response (as shown for the K^b mutants; 7). Effector cells from C57BL/6 (K^bD^b), B10.A(5R) (K^bD^d), B10.A(4R) (K^kD^b), and H-2^{bm13} (K^bD^{bm13}) mice primed with LCMV were tested on a variety of target cells that were incubated with or without the peptide GP(32 – 42). Results suggest that D^{bm13} does not present GP(32 – 42) efficiently because LCMV-infected H-2^{bm13} mice did not generate effector cells that can recognize the peptide on B10.HTG (K^dD^b) target cells bearing the relevant D^b molecule (Table 2). In the corollary assay, B10.A(4R) (K^kD^b) effector cells did not recognize peptide-coated H-2^{bm13} (K^bD^{bm13}) target cells (Table 3). These assays also showed that the K^b molecule could efficiently present the GP(32 – 42) peptide because B10.A(5R) (K^bD^d) mice lysed

Table 2. H-2^{bm13} does not mount an H-$2D^b$-restricted GP(32 – 42)-specific cytotoxic response

Effectors	Targets (% specific release)					
	MC57G (K^bD^b)		B10.A(5R) (K^bD^d)		B10.HTG (K^dD^b)	
	+	–	+	–	+	–
C57BL/6 (K^bD^b)	88	5	88	20	74	16
	71	5	64	13	54	9
	43	1	36	7	24	6
	23	3	17	4	13	3
bm13 (K^bD^{bm13})	52	15	44	7	18	17
	41	16	42	12	21	12
	24	7	25	5	8	8
	10	4	13	0	7	3

Target cells were incubated with (+) or without (–) GP(32 – 42). Spontaneous release was below 25%. Effector to target ratios shown are 70:1, 23:1, 8:1, and 3:1.

H-2^{bm13} targets incubated with GP(32 – 42) and vice versa (4) (Tables 2 and 3). These CTL assays suggest that the presentation of GP(32 – 42) by D^{bm13} is very inefficient *in vivo* because of the lack of a measurable CTL response specific for this peptide antigen.

Discussion

In this study we wanted to determine whether one particular TCR was sensitive to small changes in the MHC with respect to positive selection, negative selection, and antigen recognition. Such studies may help to determine the interactions between the TCR and its ligand during these processes and give insights into the mechanism of signalling for the different events. The transgenic LCMV/D^b-specific TCR bred into H-2^{bm13} and H-2^{bm14} mice revealed the following results. The transgenic TCR cannot be positively selected in the presence of K^b or D^{bm14}. D^{bm13} allows positive selection of the transgenic TCR, but negative selection does not occur in the presence of LCMV. The transgenic TCR cannot recognize LCMV-infected cells expressing D^{bm13}, as demonstrated by the intracerebral virus challenge, proliferation, and cytotoxicity assays (Fig. 6). The lack of recognition is consistent with the interpretation that the peptide antigen p32 – 42 is not efficiently presented by D^{bm13} and remains below a density sufficient to induce effector function or recognition *in vivo*.

Interestingly, positive selection of the D^b-restricted transgenic TCR is apparently even more efficient in the presence of the D^{bm13} allele. This was demonstrated by an increased skewing to $CD8^+$ mature thymocytes, an increased proportion of $CD3^{hi}$ thymocytes compared with the CD3 intermediate, and low subpopulations. The increased positive selection was also reflected by increased numbers of T cells in peripheral blood and lymph nodes. This increase in efficiency of positive selection is not caused by new putative selecting peptides introduced from the BALB/c background, because previous work with (C57BL/6 × BALB/c)F_1 does not show a similar increased positive selection (23). Detailed modelling studies of the D^{bm13} molecule suggest that the changes in the β-sheet of the D^{bm13} molecule should not directly alter the conformation of the class I molecule and should only affect the peptide binding (33). However, subtle changes may exist and therefore it is possible that the increase in positive selection is caused directly by the shape of the D^{bm13} MHC molecule itself. Alternatively, an altered conformation of the self peptide – MHC complex may result in an enhanced positive selection of the transgenic TCR.

Table 3. H-$2D^{bm13}$ does not efficiently present GP(32 – 42)

Effectors	Targets (% specific release)			
	MC57G (K^bD^b)		bm13 (K^bD^{bm13})	
	+	–	+	–
C57BL/6 (K^bD^b)	92	19	61	8
	86	5	75	2
	54	7	52	4
	31	1	22	5
B10.A(4R) (K^kD^b)	60	11	5	8
	34	5	5	5
	18	2	0	1
	6	2	0	4
B10.A(5R) (K^bD^d)	42	14	55	5
	43	11	49	1
	21	7	28	0
	12	4	10	0

Target cells were incubated with (+) or without (–) GP(32 – 42). Spontaneous release was below 30%. Effector to target ratios shown are 70:1, 23:1, 8:1, and 3:1.

Others have used similar approaches to analyse TCR – MHC interaction required for positive selection. Using a transgenic TCR specific for the male H-Y antigen, presented in the context of D^b, Jacobs *et al.* (18) showed that the transgenic TCR could neither be selected by H-2^{bm13} or H-2^{bm14} nor could the H-Y antigen be recognized in the context of the mutant H-2 types. A comparison with our data is interesting because both transgenic receptors are restricted to D^b, but in contrast to H-Y the LCMV-specific receptor is positively selected by D^{bm13}. This suggests that the selecting MHC complex has different requirements for different receptors. One possibility is that the conformation of a peptide presented by D^{bm13} fulfills the necessary requirements for positive selection of the LCMV-specific receptor, but not the H-Y-specific receptor.

Berg *et al.* (19) examined positive selection of a cytochrome *c* – I-E^k-restricted transgenic TCR in mice expressing I-$E_{\alpha k}$;$E_{\beta b}$.

This transgenic TCR could weakly recognize cytochrome *c* in the context of the hybrid I-E molecule, and correspondingly a weak positive selection of the transgenic TCR could also be demonstrated. In studies using a K^b-restricted transgenic TCR, Sha *et al.* (14) have also shown a reduction in positive selection in K^{bm8} mice; this mutant has substitutions on the bottom of the peptide binding groove. The authors speculated that the reduced positive selection is caused by an altered selecting peptide conformation, because the TCR should not be able to directly 'see' the changes in K^{bm8}. These data, together with the results from our transgenic D^{bm13} and D^b animals, suggest that a gradient of binding avidities exists for thymocyte selection. At one end no selection occurs and thymocytes die. A range of affinities appears to exist that results in poor to very efficient positive selection. However, if the interaction between the TCR and the MHC ligand in the thymus is too 'strong', negative selection may occur by clonal deletion. It is possible that the avidity of our TCR towards D^{bm13} may be on the border of affinities leading to either positive and/or negative selection. The increase in mature T cells in transgenic D^{bm13} mice may then be a result of peripheral expansion induced by low level stimulation of the non-deleted transgenic T cells.

The data presented here demonstrate that positive selection of the transgenic TCR is more 'efficient' in D^{bm13} animals compared with the original selecting element D^b. This suggests that the interaction between the TCR and MHC ligand in the thymus during positive selection is somewhat flexible and has different structural requirements than the TCR – peptide – MHC interactions required for negative selection or antigen recognition *in vivo*. Differences in TCR – MHC avidity may influence the ability of a given TCR to undergo positive selection and therefore may account for some of the subtle differences seen in the various TCR transgenic mouse models (23,39) and nontransgenic models (34,35).

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Abbreviations

BSS	balanced salt solution
Con A	concanavalin A
CTL	cytotoxic T lymphocyte
FCS	fetal calf serum
LCMV	lymphocytic choriomeningitis virus
Mls	mixed lymphocyte stimulating
PE	phycoerythrin
SEB	staphylococcal enterotoxin B

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